The effects of sildenafil citrate on feto–placental development and haemodynamics in a rabbit model of intrauterine growth restriction

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Abstract. The present study evaluated the effectiveness of sildenafil citrate (SC) to improve placental and fetal growth in a diet-induced rabbit model of intrauterine growth restriction (IUGR). Pregnant rabbits were fed either *ad libitum* (Group C) or restricted to 50% of dietary requirements (Group R) or restricted and treated with SC (Group SC). The treatment with SC improved placental development by increasing vascularity and vessel hypertrophy in the decidua. The assessment of feto–placental haemodynamics showed higher resistance and pulsatility indices at the middle cerebral artery (MCA) in fetuses treated with SC when compared with Group R, which had increased systolic peak and time-averaged mean velocities at the MCA. Furthermore, fetuses in the SC group had significantly higher biparietal and thoracic diameters and longer crown–rump lengths than fetuses in Group R. Hence, the SC group had a reduced IUGR rate and a higher kit size at birth compared with Group R. In conclusion, SC may provide potential benefits in pregnancies with placental insufficiency and IUGR, partially counteracting the negative effects of food restriction on placental development and fetal growth. However, the present study also found evidence of a possible blood overflow in the brain that warrants further investigation.

Introduction

The failure of fetuses to achieve their full growth potential is known as intrauterine growth restriction (IUGR). Currently, between 5 and 10% of human infants undergo IUGR (Nardozza *et al.* 2012) and, as a consequence, are at greater risk of neonatal health disorders (Maršál 2002) and late-onset diseases in adulthood (Ross and Desai 2013). The aetiology of IUGR is multifactorial and scarcely understood, but is thought to include a combination of maternal, environmental, fetal and placental factors negatively affecting fetal homeostasis (Sankaran and Kyle 2009).

The intrauterine environmental conditions of the fetus are regulated by the placenta. At present, more than 60% of IUGR offspring in developed countries are linked to abnormal placental development or placental insufficiency (Ghidini 1996). Thus, research has been focussed on devising preventive and therapeutic strategies for IUGR and, specifically, on developing therapies to improve placental development and utero–placental blood flow. An encouraging area of research is the stimulation of the placental pro-angiogenic factors placental growth factor (PIGF) and vascular endothelial growth factor (VEGF), which are primarily driven by nitric oxide (NO) and its endothelial

constitutive synthase (eNOS or NOS3). NO is a potent stimulator of vasodilatation and angiogenesis during placental development (Purcell *et al.* 1999) and decreased NO bioavailability is recognised to be involved in the pathogenesis of IUGR (Serrano *et al.* 2004). Based on this, a possible therapeutic strategy would be the administration of sildenafil citrate (SC), a vasodilator molecule that enhances NO concentrations by inhibiting phosphodiesterase-5 (PDE-5) activity (Chuang *et al.* 1998), which may optimise placental function and, therefore, alleviate IUGR in at-risk pregnancies. After preclinical studies, SC is being tested in women with promising results, improving maternal and fetal blood flow velocimetry and fetal well being (Lacassie *et al.* 2004; Lin *et al.* 2012; Panda *et al.* 2014; Sun *et al.* 2014; Trapani *et al.* 2015). Currently, several clinical trials are underway to further test the usefulness and safety of SC treatments for IUGR (Ganzevoort *et al.* 2014).

Research in human pregnancies is obviously limited by ethical and practical limitations and necessitates the use of animal models. Most studies of IUGR have been performed in rodent models (Schroder 2003). However, the rabbit is an emergent and complementary model for pregnancy studies (Eixarch *et al.* 2009; Püschel *et al.* 2010). The size of a rabbit allows serial blood sampling and imaging and also shows more similarities in metabolic, endocrine, placental and fetal features to humans than rodents (Kobayashi *et al.* 2011; Fischer *et al.* 2012; Malassiné *et al.* 2013). Specifically, the rabbit placenta is haemodichorial, which is more physiologically similar to the haemomonochorial human placenta than the haemotrichorial placenta of rodents (Fischer *et al.* 2012). Haemodynamic changes in the placenta during pregnancy in rabbits are also comparable to those in humans (Fischer *et al.* 2012; Lecarpentier *et al.* 2012), with high blood flow velocities in the umbilical arteries resembling human values in the second trimester (Polisca *et al.* 2010). Additionally, brain white-matter maturation in rabbits occurs during the perinatal period, similarly to humans, whilst in rodents this process occurs largely in the postnatal period (Beaudoin *et al.* 2003; Derrick *et al.* 2009).

Different studies in rodent models have demonstrated that SC administration during pregnancy prevents the production of inflammatory cytokines, prevents fetal loss (Luna *et al.* 2015), improves feto–placental blood flow (Stanley *et al.* 2012) and increases fetal weight (Stanley *et al.* 2012; Dilworth *et al.* 2013). However, most of the results were obtained post mortem and the ontogeny of changes in feto–placental haemodynamics and intrauterine growth are unknown.

Hence, the present study evaluated whether maternal SC administration could improve or ameliorate diet-induced defects in feto–placental development, haemodynamics and offspring outcome seen in rabbits exposed to 50% food restriction from Day 9 of pregnancy onwards, a model previously developed in our laboratory (López-Tello *et al.* 2015).

Materials and methods

Ethical approval

All experiments were carried out at the animal facilities of the Polytechnic University of Madrid (UPM, Spain), which meet the requirements of the European Union for scientific procedure establishments, under project licence of the UPM Scientific Ethic Committee. Animal manipulations were performed in accordance with the Spanish policy for animal protection RD53/2013, which complied with the European Union Directive about the protection of animals used in experimentation.

Animals and management

The experiment involved 45 New Zealand × California White rabbits (*Oryctolagus cuniculus*). Females were previously artificially inseminated and during the trial the animals were kept in individual cages under a constant photoperiod of 16 h light per day. A temperature of 18–22°C and a relative humidity of 60–75% were maintained by a forced ventilation system, according to the normal husbandry conditions for rabbits (Rebollar *et al.* 2012). All females had free access to water and were fed a diet containing 16% crude protein, 37% crude fibre, 3.7% fat and crude energy content of 2400 kcal kg^{-1} (Nanta, Spain). Daily food intake of dams was determined individually (2 weeks before starting the experiment). Food intake was measured daily by weighing the food and feeder at the beginning and at the end of the adjustment period. The mean food intake of all dams was 187.0 ± 11.0 g day^{-1}.

Experimental design

At Day 9 of pregnancy (term = Day 31), females were randomly distributed into three experimental groups. The first group was fed *ad libitum* during the entire pregnancy and considered to be the control group (Group C; $n = 15$), whilst the remaining dams were restricted individually to 50% of their average daily food intake until parturition. From Day 22 of pregnancy to delivery, half of the restricted dams were treated daily with oral SC. This was prepared by grinding Viagra tablets (100 mg; Pfizer, USA; 5 mg kg^{-1} excluding excipients) and mixing in 1 mL of baby food (Hero Baby, Spain; Group SC, $n = 15$). The remaining restricted dams received no other treatment and were considered as the untreated controls of food restriction (Group R, $n = 15$). Day 22 was chosen because it corresponds to the beginning of the period in pregnancy in which enlargement of the uterus ceases, the somatic circulation rate decreases and the incidence of IUGR is augmented due to the increase in the requirements of the fetuses for oxygen and nutrients (Reynolds 1946; López-Tello *et al.* 2015). The SC dose used in this trial was adjusted according to previous studies performed in rabbits and rats (Park *et al.* 2004; Cauli *et al.* 2010) and it was administrated orally with a syringe once per day (0900 hours) to ensure that each animal received the adequate dose, avoiding any under- or over-dosing. The use of a unique dose per day was based on the protocols of Sánchez-Aparicio *et al.* (2008) in guinea pigs and guidelines from the FDA Center for Drug Evaluation and Research in pregnant rabbits (http://www.accessdata.fda.gov/drugsatfda_docs/NDA/98/viagra/pharm_tox_pp_117_114.pdf, verified 28 April 2016). Both control groups (C and R) also received 1 mL of baby food at the same time as treated dams to avoid any possible confounding effect.

Four days after SC administration (Day 26 of pregnancy; ≈84% of the total pregnancy), four females of each group were randomly submitted to a Doppler evaluation. At Day 28 of

pregnancy ($\approx$90% of the total pregnancy), 22 dams were killed to study feto–placental morphology and the remaining females were allowed to deliver, registering data from newborns.

Study of fetuses and placentas

The dams were sedated with 35 mg kg^{-1} ketamine (Imalgene1000; Merial, Spain) and then killed using an intravenous bolus of barbiturate (30 mg kg^{-1}; Dolethal; Vetoquinol, Spain). A mid-ventral abdominal laparotomy was made to remove the entire reproductive tract. Fetuses were dissected from their extra-embryonic membranes and considered as either: (1) viable fetus (presented natural morphological features according to age and bodyweight; see Fig. S1*a*, available as Supplementary Material to this paper), (2) mummified or dead fetus (excluded from trial as we could not determinate the exact time of death; Fig. S1*b*) or (3) resorption (with atrophied fetal and maternal placenta; Fig. S1*c*).

For the viable fetuses, placentas were immediately and gently separated from the decidua (attached to the endometrium and comprised of uninucleated and giant cells in a matrix of collagen in which maternal blood passes to the implantation site through spiral arteries; Samuel *et al.* 1975) and the labyrinth (mainly composed of fetal and maternal capillaries and trophoblast responsible for nutrient and oxygen exchange; Fig. S1*d*). Both compartments were weighed and the length and thickness measured using slide calipers (values were obtained by considering the average of three consecutive measurements). Following this, fetuses were weighed and measured for crown–rump length (CRL, maximum distance from crown to tail), biparietal diameter (BPD, length from one parietal eminence to the other) and transversal thoracic diameter (TD, length at the diaphragm insertion). Fetuses were beheaded at the atlanto–occipital joint and, after cranial opening and medial laparotomy, fetal brain and liver were removed and weighed.

A viable fetus was considered to have been exposed to IUGR when its bodyweight was below the 10th percentile, assuming the control group as our standard value. Afterwards, different ratios were obtained by dividing fetal (head, brain and liver) and placental structures (decidua and labyrinth zones) by fetal weight. The weight of the brain relative to the liver was also considered as an indicator of IUGR. Finally, in order to evaluate fetal metabolic status, a total of 30 fetuses from each group were randomly selected. Blood samples were obtained after decapitation and placed in tubes with ethylenediamine tetraacetic acid (EDTA), centrifuged at 1200*g* for 10 min at 4°C to obtain plasma and immediately stored at −20°C until analysis. Parameters related to the metabolism of glucose and lipids (triglycerides and cholesterol) were measured with a clinical chemistry analyser (Saturno 300 plus; Crony Instruments, Italy) according to the manufacturer's instructions.

Placental histopathology

Sections of placentas and uteri adjacent to the ovary were collected ($n = 10$ per group), fixed in 4% paraformaldehyde for 24 h and switched to 70% ethanol for histological evaluation. Samples were embedded in paraffin, sectioned at 4-μm thickness and stained with haematoxylin–eosin following routine laboratory procedures. Sections were examined histologically by a trained pathologist blinded for the experimental procedure.

Feto–placental haemodynamics

Ultrasound scanning was carried out with a Vivid-I ultrasound machine equipped with a multi-frequency (8–12 MHz) linear array probe (General Electrics Ultraschall Deutschland GmbH, Germany). In brief, fasted animals were shaved at the abdominal area and gently restrained in dorsal recumbence, without anaesthesia to avoid any effect on heart rate and blood flow during the observations. Females usually stayed calm and relaxed during the procedure since they were regularly handled by research staff. A complete scan of the dam did not last more than 20 min. Measurements were taken from 48 fetuses (four fetuses from each female in order to minimise individual effects).

Blood-flow parameters of umbilical cord arteries (UCA) and middle cerebral arteries (MCA) were determined after identifying the vessels with colour Doppler. The waveforms of three consecutive cardiac cycles in each vessel were recorded, disregarding views with angles of insonation between 20 and 60°. Measurements were obtained after the entire examination, recording and including resistance index (RI), pulsatility index (PI), systolic peak velocity (SPV), end diastolic velocity (EDV) and time-averaged mean velocity (MV), measured at both UCA and MCA.

Neonatal study

Twenty-three dams were allowed to deliver in order to study the effects of food restriction and SC administration during pregnancy on the neonates. Immediately after birth, all the kits ($n = 251$) were classified as viable newborns ($n = 236$) or stillborns ($n = 15$). Bodyweight and morphometric parameters (CRL, BPD and TD) were measured only in viable newborns. A newborn was considered to have IUGR when its bodyweight was below the 10th percentile assuming the control group at birth as our standard value.

Statistical analysis

Statistical analyses were performed with Statistical Analysis System Software (SAS Institute Inc. Cary, NC, USA). Effects of undernutrition and sildenafil treatment on the morphological parameters of fetuses, placentas and newborns and the haemodynamic parameters of fetuses were assessed by one-way analysis of variance (one-way ANOVA); *t*-test was performed to contrast the differences between groups. The number of fetuses or kits per dam was used as a covariate. Possible differences in IUGR rate and number of placentas with histological findings were calculated by a χ^2 test. All data are reported as mean $\pm$ s.e.m. and probabilities were considered to be significant at $P < 0.05$.

Results

Morphological study of fetuses and placentas

The number of total (C, 11.95 $\pm$ 0.77; R, 12.70 $\pm$ 0.70; SC, 12.75 $\pm$ 0.75), viable (C, 11.55 $\pm$ 0.78; R, 11.60 $\pm$ 0.71; SC, 11.59 $\pm$ 0.76) and mummified (C, 0.15 $\pm$ 0.14; R, 0.22 $\pm$ 0.19; SC, 0.13 $\pm$ 0.13) fetuses and resorptions (C, 0.25 $\pm$ 0.20;

Table 1. Morphometric study of fetuses at Day 28 of pregnancy from dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. IUGR rate defined as those fetuses under 10th percentile of *ad libitum* control weights (32 g) estimated by a χ^2 test. Data represented as mean ± s.e.m. [a,b,c]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 81$)	R ($n = 94$)	SC ($n = 77$)	$P >$ f
Morphometric measurements				
Biparietal diameter (cm)	1.76 ± 0.02^{a}	1.67 ± 0.02^{b}	1.82 ± 0.02^{c}	0.001
Crown–rump length (cm)	10.52 ± 0.08^{a}	9.95 ± 0.07^{b}	10.32 ± 0.08^{a}	0.001
Thoracic diameter (cm)	1.82 ± 0.02^{a}	1.63 ± 0.02^{b}	1.70 ± 0.02^{c}	0.001
Fetus weights				
Total (g)	39.28 ± 0.64^{a}	33.91 ± 0.59^{b}	34.30 ± 0.66^{b}	0.001
Head (g)	9.46 ± 0.15^{a}	8.47 ± 0.14^{b}	8.67 ± 0.16^{b}	0.001
Body (g)	29.50 ± 0.52^{a}	25.34 ± 0.49^{b}	25.44 ± 0.54^{b}	0.001
Liver (g)	2.90 ± 0.07^{a}	2.52 ± 0.07^{b}	2.63 ± 0.08^{b}	0.001
Brain (g)	1.05 ± 0.01^{a}	0.99 ± 0.01^{b}	1.00 ± 0.01^{b}	0.001
Weight ratios				
Head weight (%)	24.22 ± 0.32^{a}	25.36 ± 0.30^{b}	25.40 ± 0.33^{b}	0.015
Brain ratio (%)	2.74 ± 0.05^{a}	3.05 ± 0.04^{b}	2.94 ± 0.05^{b}	0.001
Liver ratio (%)	7.34 ± 0.14^{a}	7.32 ± 0.13^{a}	7.74 ± 0.14^{b}	0.035
Brain : liver ratio (%)	38.66 ± 1.16^{a}	43.65 ± 1.09^{b}	39.01 ± 1.19^{a}	0.002
IUGR rate (%)	9.87^{a}	44.68^{b}	29.87^{c}	0.001

R, 0.27 ± 0.19; SC, 0.27 ± 0.20) were similar among groups. All the values for morphometric measurements were lower in Group R than in Group C ($P < 0.05$; Table 1). Conversely, the BPD of the SC group was higher than in Groups C and R ($P < 0.05$), whilst the CRL was similar to Group C and greater than Group R. For the SC group, the TD was lower than Group C but greater than Group R. Food restriction (Groups R and SC) reduced the weight of fetuses as well as the weight of head, body, liver and brain when compared with Group C ($P < 0.05$). However, SC administration was associated with an intermediate value of IUGR rate when compared with Groups C and R (Table 1 and Fig. S2). The ratios of head and brain to fetal weight (Table 1) were significantly higher in Groups R and SC ($P < 0.05$), whilst the brain to liver weight ratio was only higher in Group R ($P < 0.05$). In contrast, the ratio of liver to fetal weight was higher only in Group SC ($P < 0.05$).

With regards to metabolic status, fetuses from Group SC presented higher plasma glucose concentrations compared with Groups C and R (104.23 ± 6.72 vs 76.91 ± 8.49 and 79.35 ± 6.84 mg dL^{-1}, respectively; $P < 0.05$). However, no differences in plasma cholesterol (C, 85.17 ± 4.22; R, 81.84 ± 3.32; SC, 84.64 ± 3.28 mg dL^{-1}) or triglyceride concentrations (C, 116.90 ± 7.05; R, 116.84 ± 5.70; SC, 112.01 ± 5.87 mg dL^{-1}) were found among groups.

Maternal food restriction was also found to be related to changes in placental development in both the decidua and the labyrinth compartment (Table 2). The decidua was significantly thinner in Group R, whereas placentas in the group treated with SC had similar values to Group C. On the other hand, there was a trend for a thicker labyrinth in Group SC than in Group R ($P = 0.08$). The food restriction in both SC and R groups reduced the length of decidua and labyrinth, but did not affect compartment weight. Finally, the weight of the placenta relative to fetal weight, total placental ratio, was significantly higher in Group SC than in Group C ($P < 0.05$). The ratio between the decidua and fetal weight for Group SC showed intermediate values between Groups C and R, whilst the labyrinth to fetal weight ratio was significantly higher in Group SC when compared with the other two groups ($P < 0.05$).

Placental histopathology

Significant histological findings are summarised in Table 3 and Fig. 1. Histological changes in the placental structure were found in the junction zone and decidua region of both restricted groups (R and SC). The junction zones of a high percentage of placentas from Group R contained moderately increased amounts of poorly cellular fibrous connective tissue that extended multi-focally into the labyrinth and surrounds, and replaced vascular channels with the collapse of the adjacent labyrinth structure. Additionally, the decidua from the animals of Group R was moderately thinned when compared with the C and SC groups. On the other hand, there was a higher percentage of labyrinth and decidua samples containing moderately to markedly increased numbers of dilated small capillaries, venules and arterioles in the SC group. Interestingly, these placentas presented a higher number of and more dilated arterial sinuses compared with placentas from control and restricted animals without treatment.

Feto–placental haemodynamics

The results obtained at Day 26 of pregnancy showed a trend for higher RI ($P = 0.06$) and a significantly higher SPV ($P < 0.05$) in the UCA in both restricted groups with respect to fetuses from Group C (Table 4). There were significant effects of SC treatment on the blood flow in the fetal MCA, with fetuses in

Table 2. Placental dimensions obtained at Day 28 of pregnancy in dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. Data represented as mean ± s.e.m. IUGR rate estimated by a χ^2 test. [a,b]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 81$)	R ($n = 94$)	SC ($n = 77$)	$P > f$
Total placental weight (g)	5.62 ± 0.16	5.28 ± 0.15	5.27 ± 0.16	0.205
Total placental weight/fetal weight (%)	14.74 ± 0.41[a]	15.57 ± 0.37[ab]	16.01 ± 0.40[b]	0.030
Decidual zone				
Weight (g)	1.28 ± 0.04	1.24 ± 0.04	1.15 ± 0.04	0.100
Length (cm)	3.65 ± 0.09[a]	3.23 ± 0.09[b]	3.36 ± 0.10[b]	0.006
Thickness (cm)	0.32 ± 0.02[a]	0.26 ± 0.02[b]	0.35 ± 0.02[a]	0.001
Decidua weight/fetal weight (%)	3.41 ± 0.16[a]	3.88 ± 0.15[b]	3.49 ± 0.16[a]	0.038
Labyrinth zone				
Weight (g)	3.93 ± 0.14	3.64 ± 0.13	3.8 ± 0.14	0.323
Length (cm)	3.61 ± 0.06[a]	3.27 ± 0.05[b]	3.34 ± 0.06[b]	0.001
Thickness (cm)	0.47 ± 0.02	0.43 ± 0.01	0.48 ± 0.02	0.084
Labyrinth weight/fetal weight (%)	10.27 ± 0.34[a]	10.62 ± 0.31[a]	11.53 ± 0.34[b]	0.029

Table 3. Characteristic placental histology at Day 28 of pregnancy in dams fed *ad libitum* (C), restricted diet (R) or restricted diet treated with sildenafil citrate (SC)

Statistical analyses were performed by χ^2 test. Data represented as mean ± s.e.m. [a,b]Different superscripts within a row indicate significant differences between groups; $P < 0.05$ (number of placentas with findings compared with the total number of samples)

Parameter	C ($n = 10$)	R ($n = 10$)	SC ($n = 10$)	$P > f$
Labyrinth zone				
Collapse and fibrosis (%)	10[a] (1/10)	50[b] (5/10)	0[a] (0/10)	0.009
Junctional zone				
Fibrosis (%)	10[a] (1/10)	60[b] (6/10)	0[a] (0/10)	0.001
Increased vascularity (%)	0[a] (0/10)	0[a] (0/10)	80[b] (8/10)	0.001
Decidual zone				
Atrophy (%)	0[a] (0/10)	70[b] (7/10)	0[a] (0/10)	0.001
Hyperplastic arterial sinuses (%)	0[a] (0/10)	0[a] (0/10)	80[b] (8/10)	0.001

Group SC showing higher RI and PI than those from the other two groups ($P < 0.05$). SPV and MV measurements were also higher in the SC group than for fetuses in Group C ($P < 0.05$).

Neonatal morphological study

At parturition, no significant differences in the total number of kits delivered (C, 11.62 ± 0.88; R, 11.28 ± 1.01; SC, 9.72 ± 1.37), newborns (C, 10.62 ± 0.84; R, 10.71 ± 0.92; SC, 9.28 ± 1.25) or stillborns (C, 1 ± 0.62; R, 0.57 ± 0.30; SC, 0.42 ± 0.30) were found. Group C had the highest values for average bodyweight and morphometric measurements of BPD, CRL and TD ($P < 0.05$), whereas SC kits showed intermediate values between Groups C and R for all morphometric parameters studied except fetal weight. Food restriction significantly increased the rate of newborn IUGR ($P < 0.05$; Table 5 and Fig. S3), whilst values obtained from the group with SC administration were intermediate between Groups C and R.

Discussion

The results of the present study in a rabbit model support the usefulness of treatment with sildenafil citrate to alleviate states of placental dysfunction and improve body size in fetuses affected by IUGR.

In the present trial, sildenafil citrate therapy favoured placental growth and vascularisation, lowered IUGR incidence and resulted in offspring with increased birth size (in terms of higher values of crown–rump length and biparietal and thoracic diameters). Our results support previous data showing that maternal undernutrition during pregnancy or defects in placental development have negative effects on fetal homeostasis and give way to the appearance of IUGR (Lesage *et al.* 2001; Pardi *et al.* 2002; Matsuoka *et al.* 2006). Herein we found that a 50% reduction in maternal food intake in a rabbit model impaired placental structural phenotype (reduced length of decidua and labyrinth compartments) and led to placental pathology (such as atrophy or fibrosis). In contrast, placentas from pregnancies treated with sildenafil citrate showed significant changes when compared with those in the restricted group at the labyrinth zone (higher values of labyrinth ratio and absence of fibrosis process) and at the decidua compartment (increase in thickness and significant hyperplasia and hypertrophy of arterial sinuses). We can hypothesise that these changes are related to two main factors. First, it is known that sildenafil citrate acts as a potent angiogenesis stimulator (Pyriochou *et al.* 2007), increasing the growth of new vessels at the labyrinth (which is supported by the histological assessment, showing a higher number of small dilated capillaries when compared with untreated placentas). Concomitantly, a recent study from Luna *et al.* (2015) also found slight vasodilatation at the labyrinth zone in placentas of mice treated with this therapy. Second, the myometrium and the decidua vessels express high levels of PDE-5 (Buhimschi *et al.* 2004; Coppage *et al.* 2005) and, in the case of the rabbit, the placenta

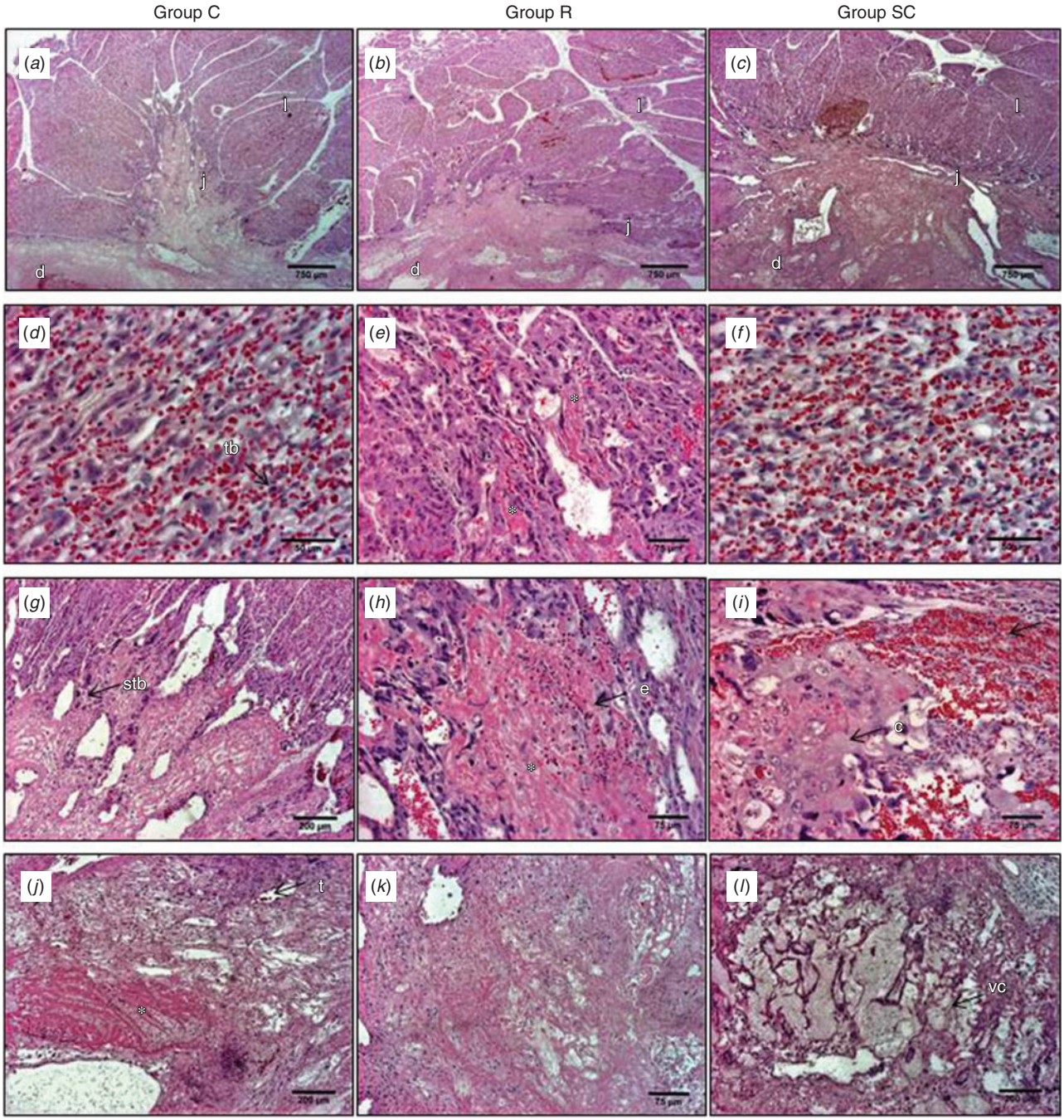

Fig. 1. Histological images of rabbit placenta at Day 28 of pregnancy in dams fed *ad libitum* (Group C), restricted diet (Group R) and restricted diet treated with sildenafil citrate (Group SC). (*a*, *b*, *c*) The three parts of the rabbit's placenta (l, labyrinth; j, junctional zone and d, decidua) in each of the three experimental groups. (*d*) Group C, normal trophoblast (tb) proliferation at the labyrinth zone. (*e*) Group R, vascular channels collapsed with multifocal areas of fibrosis (*) and mineralisation at the labyrinth zone. (*f*) Group SC, normal trophoblast proliferation at the labyrinth zone in a similar pattern to Group C. (*g*) Group C, vascularisation at the junctional zone with normal trophoblast and syncytiotrophoblasts (stb). (*h*) Group R, focus of sclerosis (*) at the junctional zone with inflammatory infiltrations (e) and decreased number of trophoblasts. (*i*) Group SC, junctional zone with syncytiotrophoblasts, increased vasculature with congestion (c) and haemorrhagic foci. (*j*) Group C, normal limits of the decidua with thrombi (t) mineral and inflammation (*). (k) Group R, decidua with necrosis, fibrin and few vascular channels. (*l*) Group SC, decidua with numerous dilated vascular channels (vc).

Table 4. Feto–placental haemodynamics at Day 26 of pregnancy from dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. Data represented as mean ± s.e.m. [a,b]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 16$)	R ($n = 16$)	SC ($n = 16$)	$P > f$
Umbilical cord arteries				
Resistance index	0.70 ± 0.02	0.77 ± 0.01	0.77 ± 0.05	0.066
Pulsatility index	1.20 ± 0.05	1.25 ± 0.04	1.28 ± 0.05	0.468
Systolic peak velocity	33.20 ± 4.05[a]	42.60 ± 3.46[b]	43.90 ± 3.35[b]	0.012
End diastolic velocity	8.00 ± 0.86	10.30 ± 1.16	10.30 ± 1.15	0.336
Time-averaged mean velocity	20.60 ± 2.37	26.40 ± 2.25	27.10 ± 2.17	0.137
Middle cerebral artery				
Resistance index	0.60 ± 0.02[a]	0.60 ± 0.02[a]	0.70 ± 0.02[b]	0.023
Pulsatility index	0.90 ± 0.05[a]	0.90 ± 0.05[a]	1.10 ± 0.05[b]	0.013
Systolic peak velocity	17.10 ± 2.21[a]	22.20 ± 1.94[b]	26.30 ± 2.02[b]	0.015
End diastolic velocity	6.50 ± 0.63	8.30 ± 0.78	7.70 ± 0.46	0.165
Time-averaged mean velocity	11.80 ± 1.40[a]	15.30 ± 1.28[b]	17.00 ± 1.13[b]	0.026

Table 5. Morphometric measurements of newborns from dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. Data represented as mean ± s.e.m. IUGR rate defined as those fetuses under 10th percentile of *ad libitum* control weights (37.9 g) estimated by a χ^2 test. [a,b,c]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 85$)	R ($n = 75$)	SC ($n = 76$)	$P > f$
Bodyweight (g)	55.23 ± 1.11[a]	49.46 ± 1.18[b]	47.80 ± 1.19[b]	0.001
Biparietal diameter (cm)	2.20 ± 0.01[a]	2.08 ± 0.01[b]	2.15 ± 0.01[c]	0.001
Crown–rump length (cm)	11.00 ± 0.09[a]	10.20 ± 0.09[b]	10.50 ± 0.10[c]	0.001
Thoracic diameter (cm)	2.40 ± 0.02[a]	2.17 ± 0.02[b]	2.28 ± 0.02[c]	0.001
IUGR rate (%)	9.41[a]	38.66[b]	25.00[c]	0.001

has a high expression level of NOS, especially NOS3 (Khan *et al.* 2012), which may facilitate sildenafil citrate function. Both processes would be expected to stimulate neoangiogenesis and may also improve maternal blood flow to the placenta, thereby facilitating nutrient and oxygen delivery to the fetus.

Consequently, fetal growth was improved in terms of crown–rump length and biparietal and thoracic diameters, at Day 28 and at birth. These findings support previous results from Sánchez-Aparicio *et al.* (2008) and Stanley *et al.* (2012). However, this larger body size was not concomitant with increases in fetal weight, supporting data from previous studies in rodent models (Ramesar *et al.* 2010; George *et al.* 2013; Motta *et al.* 2015). Notwithstanding, studies in sheep with 50% food restriction (similar to our restriction) have shown that sildenafil citrate treatment increased fetal weight by 14% (Satterfield *et al.* 2010). Such disagreement may be related to differences in nutrient partitioning between monotocous and polytocous species (Fowden and Moore 2012), the capacity of the placenta to adapt its phenotype or function to undernutrition and the length of sildenafil citrate therapy (a total of 87 days, from Day 28 to Day 115 of pregnancy, Satterfield *et al.* 2010).

A remarkable finding of this novel study using a rabbit model comes from the results obtained by assessing the relative growth of fetal organs with respect to fetal weight, which may set the basis for future studies on the use of sildenafil citrate therapies and its impact on fetal organs. Fetuses treated with sildenafil citrate developed a proportionally larger liver with respect to the other two groups. This observation is in line with results obtained in rats (Pellicer *et al.* 2011). It is well known that from early development the liver is vital for health and body physiology. It participates in fat deposition (Godfrey *et al.* 2012), regulates growth and metabolism by modulating hormones and growth factors (Hellerstein and Munro 1994; Tchirikov *et al.* 2002) and is responsible for gluconeogenesis (Burns *et al.* 1997), the latter of which could explain the high glucose level found in the SC group. But, even more importantly, the liver can also modulate blood distribution as it is the first organ to receive blood from the placenta (Tchirikov *et al.* 2002) and, due to the presence of the ductus venosus, may distribute blood towards essential organs at the expense of less-essential organs (Cohn *et al.* 1974; Jensen *et al.* 1991). As a result of alterations in the liver, blood distribution may have changed, favouring developmental adaptation in the fetus by selectively increasing blood flow to vital organs like the brain (‘brain-sparing effect’).

The data obtained in the present study support the idea that undernutrition early in pregnancy may affect vital organs leading to disproportionate growth of the fetus (Bauer *et al.* 2003; Desai *et al.* 2007) and that the fetus can counteract this by an innate mechanism of fetal cardiac output distribution (Giussani 2011). In the present study, restricted fetuses showed higher head and brain mass relative to bodyweight, which suggests asymmetric growth retardation of the fetus, supporting the idea of the ‘thrifty phenotype’ (Wells 2011). Also, these data support previous studies in which the comparison of the brain weight to liver weight ratio was associated with undernutrition and dysmaturity (Anderson 1972; Camm *et al.* 2010). However,

the results obtained by dividing these weights suggest that therapy with sildenafil citrate could ameliorate this ratio. Another finding in this study is that, although this brain-sparing effect has been proposed to be stimulated by the hypoglycaemic status in the restricted fetus (Giussani 2011), data obtained in this rabbit model suggest that there may be factors in addition to fetal glycaemic status that contribute.

Assessment of blood flow by Doppler ultrasonography at Day 26 of pregnancy showed that food restriction induced changes in the haemodynamic patterns of the fetus. Those IUGR fetuses exhibited a trend to increase the umbilical artery resistance index and demonstrated a significant increase in the systolic peak velocity, suggesting a deterioration of placental function (Carr *et al.* 2012). These blood-flow changes could not be rescued by sildenafil citrate, which is contrary to data from previous studies (Dastjerdi *et al.* 2012; Lin *et al.* 2012; Stanley *et al.* 2012; Trapani *et al.* 2015). As a consequence of the placental dysfunction and reduction in oxygen levels, the middle cerebral artery in both restricted and sildenafil-treated fetuses exhibited changes in the systolic mean velocity and therefore increased mean velocity values, which agrees with previous data on the middle cerebral artery of fetuses affected by IUGR (Hanif *et al.* 2007; Mari *et al.* 2007).

Nevertheless, fetuses undergoing sildenafil citrate therapy showed elevated values of pulsatility and resistance indexes, as Dastjerdi *et al.* (2012) found in pregnant women, which may suggest a certain grade of vasoconstriction (low indices reflects redistribution of cardiac output to the brain; Mari *et al.* 2007). It is known that cerebral neurons and vessels have high concentrations of PDE-5 (Kotera *et al.* 2000; Lin *et al.* 2006) and that sildenafil citrate can cross the placenta (Pellicer *et al.* 2011). Moreover, this therapy can increase brain cGMP levels (Zhang *et al.* 2002) and cerebral blood flow (Li *et al.* 2007). Taken together, these data suggest that fetuses from sildenafil-treated mothers activate a protective mechanism in the middle cerebral artery to counteract an excess in blood-flow supply that could produce cerebral oedema and consequent adverse neurological outcomes. However, these data should be interpreted with caution, as the Doppler assessment was only performed once during the pregnancy, and thus other possible changes in gestation could not be determined in response to sildenafil citrate in this study. Further studies are needed to determine the possible risks of blood overflow in the fetal brain and also to identify the mechanism by which the fetus is able to adapt its cerebral arterial vascular tone; this process possibly depends on the enhancement of nitric oxide abundance with sildenafil citrate administration. Furthermore, elucidating whether these haemodynamic and morphologic adaptations of the fetus can have consequences in adult life should be the focus of future investigations.

In summary, the results of the present study suggest that, in rabbits, a 50% restriction of maternal food intake is a valid model for inducing IUGR and placental insufficiency. Such effects can be partially counteracted by the administration of sildenafil citrate, since it improves reductions in perinatal body size and modifies placental growth and vascularisation in the labyrinth and decidua. Therefore, size of the newborns can be partially improved. Thus, our study sets the basis of further studies investigating the use of PDE-5 inhibitors to study organ development and the programming of offspring growth and postnatal homeostasis (in particular brain and liver function).

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